

**THIS PAGE IS INSERTED BY OIPE SCANNING
AND IS NOT PART OF THE OFFICIAL RECORD**

Best Available Images

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

BLACK BORDERS ✓

TEXT CUT OFF AT TOP, BOTTOM OR SIDES

FADED TEXT

BLURRY OR ILLEGIBLE TEXT

SKEWED/SLANTED IMAGES

COLORED PHOTOS HAVE BEEN RENDERED INTO BLACK AND WHITE

VERY DARK BLACK AND WHITE PHOTOS

UNDECIPHERABLE GRAY SCALE DOCUMENTS

**IMAGES ARE THE BEST AVAILABLE
COPY. AS RESCANNING *WILL NOT*
CORRECT IMAGES, PLEASE DO NOT
REPORT THE IMAGES TO THE
PROBLEM IMAGE BOX.**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68	A2	(11) International Publication Number: WO 93/24657 (43) International Publication Date: 9 December 1993 (09.12.93)
(21) International Application Number: PCT/US93/04946 (22) International Filing Date: 25 May 1993 (25.05.93) (30) Priority data: 07/890,719 29 May 1992 (29.05.92) US 08/049,678 16 April 1993 (16.04.93) US (71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors: BLUMENFELD, Anat ; 211 Mason Terrace, Brookline, MA 02176 (US). GUSELLA, James, F. ; 7 Woodstock Drive, Framingham, MA 01701 (US). BREAKFIELD, Xandra, O. ; 127 Homer Street, Newton, MA 02159 (US).		(74) Agents: RZUCIDLO, Eugene, C. et al.; Morgan & Finnegan, 345 Park Avenue, New York, NY 10154 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DE (Utility model), DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: USE OF GENETIC MARKERS TO DIAGNOSE FAMILIAL DYSAUTONOMIA (57) Abstract <p>Familial dysautonomia (FD), the Riley-Day syndrome, is an autosomal recessive disorder characterized by developmental loss of neurons from the sensory and autonomic nervous system. It is limited to the Ashkenazi Jewish population, where the carrier frequency is 1 in 30. We have mapped the FD gene to the chromosome region 9q31-q33 by linkage with ten DNA markers in twenty-six families. The maximum lod score of 21.1 with no recombinants was achieved with <i>D9S58</i>. This marker also showed strong linkage disequilibrium with FD, with one allele present on 73 % of all affected chromosomes compared to 5.4 % of control chromosomes ($X^2 = 3142$, 15 d.f. $p < 0.0001$). The other nine markers, distributed within 23 cM proximal or distal to <i>D9S58</i>, also yielded significant linkage to FD. <i>D9S53</i> and <i>D9S105</i> represent the closest flanking markers for the disease gene. This localization will permit prenatal diagnosis of FD in affected families.</p> <div data-bbox="760 1203 1422 1843"><div>Genetic map</div><div>Physical location</div></div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

- 1 -

USE OF GENETIC MARKERS TO DIAGNOSE FAMILIAL DYSAUTONOMIA

5 This application is a continuation-in-part of
United States Patent Application 07/890,719 filed May 29,
1992.

10

STATEMENT AS TO RIGHTS TO INVENTION

 The present invention was developed at
Massachusetts General Hospital under obligation to assign
the invention to the same. The Dysautonomia Foundation has
15 an option for an exclusive license for the present
invention.

20

FIELD OF THE INVENTION

 This invention relates to genetic testing; more
specifically, to a method of detecting the presence of the
familial dysautonomia gene and also the identification of
25 the location of the familial dysautonomia gene in the
genome.

30

BACKGROUND OF THE INVENTION

 Familial dysautonomia, or the Riley-Day syndrome,
is a rare inherited neurological disease affecting the
development and survival of sensory, sympathetic and some
parasympathetic neurons (Riley, C.M. et al., Pediatrics
35

- 2 -

1949; 3:468-477; Axelrod, F.B., et al., Am. J. Dis. Child.
1984; 138:947-954; Axelrod, F.B., Cell. Molec. Biol.
Neuronal Dev. 1984; Ed.: Black, I.B., Plenum Press, NY; 331-
340). It is the most common and the best known of a group
5 of rare disorders, termed congenital sensory neuropathies,
that are characterized by widespread sensory, and variable
autonomic dysfunction. Patients with familial dysautonomia
are affected from birth with a variety of symptoms such as
10 decreased sensitivity to pain and temperature, vomiting
crises and cardiovascular instability all of which might
result from a deficiency in a neuronal growth factor pathway
(Breakefield, X.O., et al., Proc. Natl. Acad. Sci. USA 1984;
15 81:4213-4216; Breakefield, X.O., et al., Mol. Biol. Med.
1986; 3:483-494). Neuropathological findings have clearly
differentiated familial dysautonomia from other congenital
sensory neuropathies (Axelrod, F.B., et al., Am. J. Dis.
20 Child., supra, Axelrod, F.B., Cell. Molec. Biol. Neuronal
Dev., supra.) The disorder is inherited as an autosomal
recessive with complete penetrance and is currently confined
25 to individuals of Ashkenazi Jewish descent (Brunt, P.W., et
al., Medicine 1970; 49:343-374). In this population, the
estimated carrier frequency is 1 in 30 with a disease
incidence of 1 in 3600 births (Maayan, C., et al., Clinical
30 Genet. 1987; 32:106-108). The clear-cut pattern of
transmission, apparent restriction to one ethnic population
and lack of confounding phenocopies suggest that all cases
of familial dysautonomia might have descended from a single
35

- 3 -

mutation (Axelrod, F.B., et al., Am. J. Dis. Child., supra,
Axelrod, F.B., Cell. Molec. Biol. Neuronal Dev., supra).

For more than 40 years, familial dysautonomia
related research concentrated on biochemical, physiological
5 and histological-pathological aspects of the disorder.
Although those studies contributed to a better understanding
of the nature of the disease, and indicated that a
deficiency in a neuronal growth factor pathway might be the
10 cause of familial dysautonomia, they did not result in
identification of the familial dysautonomia gene, thus,
those studies did not contribute to the availability of a
genetic test for familial dysautonomia.

15 Chromosomal localization of the gene causing
familial dysautonomia can facilitate genetic counseling and
prenatal diagnosis in affected families. Subsequent
20 delineation of closely linked markers which show strong
linkage disequilibrium with the disorder and ultimately,
identification of the defective gene can allow screening of
the entire at-risk population to identify carriers, and
25 potentially reduce the incidence of new cases.

Linkage analysis can be used to find the location
of a gene causing a hereditary disorder and does not require
any knowledge of the biochemical nature of the disease, i.e.
30 the mutated protein that is believed to cause the disease.
Traditional approaches depend on assumptions concerning the
disease process that might implicate a known protein as a
candidate to be evaluated. The genetic localization
35

- 4 -

approach using linkage analysis (positional cloning) can be used to first find the chromosomal region in which the defective gene is located and then to gradually reduce the size of the region in order to determine the location of the specific mutated gene as precisely as possible. After the gene itself is identified within the candidate region, the messenger RNA and the protein are identified and along with the DNA, are checked for mutations.

This latter approach has practical implications since the location of the gene can be used for prenatal diagnosis even before the altered gene that causes the disease is found. Identification of DNA markers linked to the disease gene will enable molecular diagnosis of carriers of the disease gene for familial dysautonomia and the determination of the probability of having the disease. This identification of the presence of the disease gene also enables persons to evaluate either genetic probability of passing this gene to their offspring or the presence of the mutated gene in an unborn child. The mutation(s) in the specific gene responsible for the pathogenesis of familial dysautonomia has its origin in the Ashkenazi Jewish population. Accordingly, individuals of Ashkenazi Jewish descent are at greatest risk of carrying the altered gene.

The transmission of a disease within families, then, can be used to find the defective gene. This approach to molecular etiology is especially useful in studies of inherited neurological disorders, as only several thousands of

35

- 5 -

the hundred-or-so thousand genes active in the nervous system are known, and nervous tissue is hard to obtain for biochemical analysis.

Linkage analysis is possible because of the nature of inheritance of chromosomes from parents to offspring. During meiosis the two homologues pair to guide their proper separation to daughter cells. While they are lined up and paired, the two homologues exchange pieces of the chromosomes, in an event called "crossing over" or "recombination". The resulting chromosomes are chimeric, that is, they contain parts that originate from both parental homologues. The closer together two sequences are on the chromosome, the less likely that a recombination event will occur between them, and the more closely linked they are. In a linkage analysis experiment, two positions on a chromosome are followed from one generation to the next to determine the frequency of recombination between them. In a study of an inherited disease, one of the chromosomal positions is marked by the disease gene or its normal counterpart, i.e. the inheritance of the chromosomal region can be determined by examining whether the individual displays symptoms of the disorder or is a parent of an affected individual (carrier) or not. The other position is marked by a DNA sequence that shows natural variation in the population such that the two homologues can be distinguished based on the copy of the "marker" sequence that they possess. In every family, the inheritance of the genetic

- 6 -

marker sequence is compared to the inheritance of the disease state. If within a family carrying a recessive disorder such as familial dysautonomia every affected individual carries the same form of the marker and all the unaffected individuals carry at least one different form of the marker, there is a great probability that the disease gene and the marker are located close to each other. In this way, chromosomes may be systematically checked with known markers and compared to the disease state. The data obtained from the different families is combined, and analyzed together by a computer using statistical methods. The result is information indicating the probability of linkage between the genetic marker and the disease gene at different distance intervals. A positive result indicates that the disease is very close to the marker, while a negative result indicates that it is far away on that chromosome, or on an entirely different chromosome.

Linkage analysis is performed by typing all members of the affected family at a given marker locus and evaluating the co-inheritance of a particular disease gene with the marker probe, thereby determining whether the two of them are close to each other in the genome. The recombination frequency can be used as a measure of the genetic distance between two gene loci. A recombination frequency of 1% is equivalent to 1 map unit, or 1 centiMorgan (cM), which is roughly equivalent to 1,000 kb of

- 7 -

- DNA. This relationship holds up to frequencies of about 20% (or 20 cM).

The entire human genome is 3,300 cM long. In order to find an unknown disease gene within 5-10 cM of a marker locus, the whole human genome can be searched with 165-330 informative marker loci spaced at 5-10 cM intervals (Botstein, D.R.I., et al., Am. J. Hum. Genet. 1980; 32:314-331). The reliability of linkage results is established by using a number of statistical methods.

The method most commonly used for the analysis of linkage in humans is the LOD score method, developed by Morton, 1955; and incorporated into the computer program LIPED by Ott, 1976. LOD scores are the logarithm of the ratio of the likelihood that two loci are linked at a given distance to that they are not linked (>50 cM apart). The advantage of using logarithmic values is that they can be summed among families with the same disease. This becomes necessary given the relatively small size of human families.

By convention, a total lod score greater than +3.0 (that is, odds of linkage at the specified recombination frequency being 1000 times greater than odds of no linkage) is considered to be significant evidence for linkage at that particular recombination frequency; a total lod score of less than -2.0 (that is, odds of no linkage being 100 times greater than odds of linkage at the specified frequency) is considered to be strong evidence that the two loci under

- 8 -

consideration are not linked at that particular recombination frequency.

Until recently, most linkage analyses have been performed on the basis of two-point data; that is, the relationship between the disorder under consideration and a particular genetic marker. However, as a result of the rapid advances in mapping the human genome over the last few years, and concomitant improvements in computer methodology, it has become feasible to carry out linkage analyses using multi-point data; that is, a simultaneous analysis of linkage between the disease and several linked genetic markers, when the recombination (genetic) distance among the markers is known.

Multi-point analysis is advantageous for two reasons. First, the informativeness of the pedigree is usually increased. Each pedigree has a certain amount of potential information, dependent on the number of parents heterozygous for the marker loci and the number of affected individuals in the family. However, not all markers are sufficiently polymorphic as to be informative in all those individuals. If multiple markers are considered simultaneously, then the probability of an individual being heterozygous for at least one of the markers is greatly increased. Second, and more important, an indication of the position of the disease gene among the markers may be determined. This may allow identification of flanking markers, and thus eventually allows isolation of a small

- 9 -

region in which the disease gene resides. Lathrop, G.M., et al., Proc. Natl. Acad. Sci. USA 1984; 81:3443-3446 have written the most widely used computer package, LINKAGE, for multi-point analysis.

5 When two loci are extremely close together, recombination between them is very rare. In fact, the rate at which the two neighboring loci recombine can be so slow as to be unobservable except over many generations. The
10 resulting allelic association is generally referred to as linkage disequilibrium. Linkage disequilibrium is defined as specific alleles at two loci that are observed together
15 on a chromosome more often than expected from their frequencies in the population. Such results are strongly influenced by founder and subpopulation effects, so it is generally necessary to examine data only within one ethnic
20 group or population isolate, which is the case for familial dysautonomia, which is only found in individuals of Ashkenazi Jewish descent. Linkage disequilibrium is usually used to further define the chromosomal region containing the
25 disease gene, once linkage has been demonstrated in a specific region. When disequilibrium is suspected, the affected individuals are checked for increased frequency of specific alleles for the marker loci. An excess frequency
30 of any allele, as measured against general population frequencies (using the Chi-square statistics) would indicate linkage disequilibrium. The major advantage of
35 disequilibrium study over standard linkage analysis is the

- 10 -

- need to test only a single affected individual per family, which is the usual case with rare recessive disorders, thus increasing the population amenable for analysis.

The marker locus must be very tightly linked to the disease locus in order for linkage disequilibrium to exist. Potentially, markers within a few cM of the disease gene could be examined and no linkage disequilibrium detected. Linkage disequilibrium has been observed with markers within 500 kb of the cystic fibrosis gene (Kerem, et al., Science 1989; 245:1073-1080). If linkage is found with several marker loci that are spaced along several centiMorgans, and none of them show recombination between the marker tested and the disease status in affected families, disequilibrium is the only genetic approach that can narrow down the chromosomal region linked to the disease gene.

A specific DNA sequence in an individual can undergo many different changes, such as deletion of a sequence of DNA, insertion of a sequence that was duplicated, inversion of a sequence, or conversion of a single nucleotide to another. Changes in a specific DNA may be traced by using restriction enzymes that recognize specific DNA sequences of 4-6 nucleotides. Restriction enzymes, cut (digest) the DNA at their specific recognized sequence, resulting in one million or so pieces. When a difference exists that changes a sequence recognized by a restriction enzyme to one not recognized, the piece of DNA

- 11 -

produced by cutting the region will be of a different size. The various possible fragment sizes from a given region therefore depend on the precise sequence of DNA in the region. Variation in the fragments produced is termed
5 "restriction fragment length polymorphism" (RFLP). The different sized-fragments reflecting different variant DNA sequences can be visualized by separating the digested DNA according to its size on an agarose gel and visualizing the
10 individual fragments by annealing to a radioactively labeled, DNA "probe". Each individual can carry two different forms of the specific sequence. When the two homologues carry the same form of the polymorphism, one band
15 will be seen. More than two forms of a polymorphism may exist for a specific DNA marker in the population, but in one family just four forms are possible; two from each parent. Each child inherits one form of the polymorphism
20 from each parent. Thus, the origin of each chromosome can be traced (maternal or paternal origin).

RFLPs have proven to be somewhat limiting in that
25 they usually give only two alleles at a locus and not all parents are heterozygous for these alleles and thus informative for linkage. Newer methods take advantage of the presence of DNA sequences that are repeated in tandem,
30 variable numbers of time and that are scattered throughout the human genome. The first of these described were variable number tandem repeats of core sequences (VNTRs)
(Jeffreys, A.J.V., et al., Nature 1985; 314:67-73; Nakamura,
35

- 12 -

Y.M., et al., Science 1987; 235:1616-1622). VNTRs are detected using unique sequences of DNA adjacent to the tandem repeat as marker probes, and digesting the DNA with restriction enzymes that do not recognize sites within the core sequence. However, highly informative VNTR loci have not been found on all chromosome arms, and those which have been identified are often situated near telomeres (Royle, et al., Genomics 1988; 3:352-360), leaving large regions of the genome out of reach of these multiallelic marker loci.

Recently, it was discovered that eukaryotic DNA has tandem repeats of very short simple sequences termed SSRs (Simple Sequence Repeat polymorphisms) such as (dC-dA)_n(dG-dT)_n where n = 10-60 (termed GT repeat). The (dG-dT) repeats occur every 30-60 kb along the genome (Weber, J.L., et al., Am. J. Hum. Genet., 1989; 44:388-396; Litt, M., et al. Am. J. Hum. Genet., 1989; 44:397-401), and Alu 3' (A)_n repeats occur approximately every 5 kb (Economou, Proc. Natl. Acad. Sci. U.S.A. 1990; 87:2951-4). Other repeats, such as GA repeats, trinucleotide and tetranucleotide repeats are less common.

Oligonucleotides encoding flanking regions of these repeats are used as primers for the polymerase chain reaction (PCR) (Saiki, Science 1988; 239:484-491) on a small sample of DNA. By amplifying the DNA with radioactive nucleotides, the sample may be quickly resolved on a sequencing gel and visualized by autoradiography. Because these polymorphisms are comprised of alleles differing in

- 13 -

- length by only a few base pairs, they are not detectable by conventional Southern blotting as used in traditional RFLP analysis.

The use of PCR to characterize SSRs such as GT polymorphic markers enables the use of less DNA, typically only ten nanograms of genomic DNA is needed, and is faster than standard RFLP analysis, because it essentially only involves amplification and electrophoresis (Weber, supra).

Consequently, the present invention comprises genetic linkage analysis to identify an individual having the familial dysautonomia gene. In addition, discovery of markers linked to the familial dysautonomia gene will enable researchers to focus future analysis on a small chromosomal region and will accelerate the identification and sequencing of the familial dysautonomia gene.

It is an object of the present invention to locate markers linked to the familial dysautonomia gene and to identify the location of the familial dysautonomia gene in the human genome.

It is a further object of the present invention to provide a genetic test specific for the familial dysautonomia gene by analysis of DNA markers linked to the familial dysautonomia gene.

It is a still further object of the present invention to provide a genetic test for the prenatal diagnosis and carrier detection specific for the familial

35

- 14 -

- dysautonomia gene by analysis of DNA markers linked to the familial dysautonomia gene.

It is a further object of the present invention to isolate and characterize the gene for familial dysautonomia by analysis of DNA markers linked to the familial dysautonomia gene.

SUMMARY OF THE INVENTION

The present invention describes, for the first time, the chromosomal location which carries the gene responsible for familial dysautonomia and provides a method of detecting the presence of a familial dysautonomia gene in a subject. The location by applicants of the familial dysautonomia gene is on the long arm of human chromosome 9 (q arm) more specifically between *D9S59* and *D9S127*. In addition, we have mapped the FD gene to the chromosome region 9q31-q33. Within the chromosomal region defined by *D9S59* and *D9S127*, the closest flanking markers for the disease gene are *D9S53* and *D9S105*. A most probable location of the familial dysautonomia gene is close to *D9S58*.

Linkage analysis with markers located on the long arm of human chromosome 9 is used to identify the inheritance of the allele causing familial dysautonomia with 80-90% accuracy, or greater, at the present time.

In particular, the test is carried out by studying the heritability of a combination of two or more polymorphisms linked to familial dysautonomia among any

- 15 -

- number of suitable family members so as to allow the determination of phenotype. The test can be used prenatally to screen a fetus or presymptomatically, to screen a subject at risk through his/her family.

5 The invention also extends to products useful for carrying out the assay, such as DNA probes (labelled or unlabelled), kits and the like.

10

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 -- Pedigrees of twenty-six families affected with familial dysautonomia that were used for linkage analysis. Symbols: empty circle unaffected female; filled circle, affected female; empty square, unaffected male; filled square, affected male; slashed symbol, deceased; star symbol, blood not collected.

20

FIGURE 2 -- Table of lod scores of different chromosome 9 markers in dysautonomia families. The lod scores were calculated assuming conventional recombination values (θ) between familial dysautonomia and the marker; 0, 0.05, 0.1, 0.2, 0.3, 0.4. When there is at least one recombination event between a marker tested and the disease, the lod score at $\theta=0$ is minus infinity. At other recombination values, lod scores can be positive or negative. The highest lod score obtained by each marker (\hat{Z}), and the recombination value in which that lod score was calculated ($\hat{\theta}$), are also included. This gives a rough

35

- 16 -

estimation of the genetic distance between the marker and the disease. The markers are ordered according to their location on chromosome 9, when *D9S15* is the most centromeric one, and *ASS* is the closest to the telomere. In some cases, the order of the markers is unknown, because they were not placed on the same genetic map and were not typed with the same pedigrees (*D9S109-D9S29-D9S127*). In this case the order was determined according to $\hat{\theta}$ and according to recombination events that were detected while setting the phase (maternal or paternal origin) of the chromosomes in FD families. Additional data from other markers is also presented in Table 2.

FIGURE 3 -- Physical and genetic map of chromosome 9 markers.

Physical map of human chromosome 9 markers. The names of the bands on chromosome 9 were determined according to Giemsa dyes. All the markers that show linkage with familial dysautonomia (Fig. 2) are located on the long arm (q arm) of chromosome 9, most of which are on band 31.

The genetic map positions of those FD-linked markers whose relative order was supported by odds of greater than 1000:1 was determined from combining the CEPH panel and the Venezuelan reference pedigree. The relative order of *D9S109* and *D9S127* could not be determined. *D9S29* could not be positioned, but data from the FD families suggest that it maps as shown.

- 17 -

• **FIGURE 4 -- Examples of recombination events localizing FD.**

The region cosegregating with FD is shown as a filled box in two nuclear families (A: pedigree 21; B: pedigree 17). Hatched boxes indicate uncertainty with respect to the precise position of a crossover due to unformativeness of *D9S58* in the mother of pedigree 21, and *D9S109* in the mother of pedigree 17. The recombination event in pedigree 21 is the only instance of all 26 FD families where a crossover occurred within the *D9S53-D9S105* interval that could not be placed relative to *D9S58*.

15 **FIGURE 5 -- Multipoint linkage of FD to chromosome 9.**

FD was mapped with respect to the following map generated from the Venezuelan reference pedigree:

D9S53-7.5cM -D9S58 - 3.1cM - D9S105 1.7cM - D9S59

20 Arrows denote the map location of each marker locus.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 The present invention further describes, for the first time, the location and chromosomal band which carries the gene responsible for familial dysautonomia.

To find the chromosomal location of the familial dysautonomia gene, polymorphic markers were typed in 26 families (Fig. 1). All selected families have two or three dysautonomic members (25 families), or consanguinity between the parents (one family). Thirty other families with one surviving FD member and 125 patients whose parents were not

- 18 -

collected were used for allelic association studies. All families were collected in North America or Israel. The diagnosis of FD was confirmed in all cases based on standard criteria (Axelrod, F.B. & Pearson, J., Am. J. Dis. Child. 1984; 138:947-954; Axelrod, F.B., Cell. and Molec. Biol. of Neuronal Dev. 1984; Ed: Black IB Plenum Press, NY 331-340). DNAs from all family members, carriers, affected and unaffected, were tested with each marker. The result of the typing was compared to the disease status of each individual. Linkage analysis computer data management and statistical programs were employed and the lod scores of the different families were pooled together to give the lod score for each marker at different distances from the disease.

Control individuals were unrelated members of Ashkenazi Jewish families with idiopathic torsion dystonia (n=130, 260 chromosomes) who manifested no dystonic or dysautonomic symptoms. The torsion dystonia gene (DYTI) was mapped to 9q34 (Kramer, P.L. et al., Ann. Neurol. 1990; 27:114-120); and is tightly linked to ABL and ASS, (Ozelius, L.J. et al., Am. J. Hum. Genet 1992; 50:619-628); both of which were excluded for linkage with FD (Table 2). Unaffected chromosomes from the FD parents (110 chromosomes) were not used in the linkage disequilibrium studies; however, they yielded allele frequencies similar to the other control population.

- 19 -

Over 250 DNA polymorphisms distributed on all 22 autosomes were checked before linkage was found. Most of the polymorphisms yielded negative lod scores values and, hence, allowed the exclusion of chromosomal regions as possible sites for the familial dysautonomia gene. The few that had positive lod scores at some distances from the markers, were slightly positive but far from +3.0, that is conventionally accepted as a minimal demand for linkage. Moreover, those slightly positive markers were surrounded by negative flanking markers, indicating that the familial dysautonomia gene was not in the immediate vicinity of that chromosomal region.

The present invention relates to the location of polymorphic markers on the long arm of human chromosome 9, which are linked to the familial dysautonomia gene and enables linkage analysis to predict both an affected individual having both familial dysautonomia genes and a carrier with only one familial dysautonomia gene. Linkage analysis with these polymorphisms can identify the inheritance of the familial dysautonomia allele with at least 80-90% accuracy. Polymorphisms are DNA sequences located on the long arm of human chromosome 9. More specifically those polymorphisms are in, or immediately adjacent to the q31 band on the long arm of chromosome 9. Even more specifically, the familial dysautonomia gene is mapped to the chromosome region 9q31-q33 by linkage with ten DNA markers in twenty-six families. The linkage analysis of

- 20 -

the invention can be carried out by using any polymorphism linked to the familial dysautonomia allele. The use of the term polymorphism is intended to encompass any marker DNA sequence which is linked to the familial dysautonomia gene.

5 The polymorphism can be a polymorphic repeating sequence or allelic forms of a gene. The polymorphism must be located close to, or be the same as, the familial dysautonomia gene. If located close to the familial dysautonomia gene, the

10 polymorphism must be sufficiently close to the familial dysautonomia gene such that the familial dysautonomia gene and the marker are linked. Linkage may be determined by a

15 significant lod score or other acceptable statistical linkage determination.

The marker can be detected by a variety of methods. The preferred detection means uses radioactive

20 nucleotides in PCR amplification of the polymorphism, or randomly labeled probes in hybridization reactions. Other detection methods such as the ligase chain reaction (LCR) can also be used. The polymorphism can be detectably

25 labeled by a radioisotope or by chemical modification enabling direct detection of the polymorphism. Fluorescent or colorimetric means can also be used. Detection of the

30 polymorphism can be indirect, e.g. a radioactive complementary strand of DNA, resulting from incorporation of radioactive nucleotides in a polymerase chain reaction.

For typing restriction fragment length

35 polymorphisms (RFLPs) and VNTR polymorphisms, genomic DNA

- 21 -

prepared from cell lines derived from all members of families affected with familial dysautonomia was digested with restriction endonuclease, resolved by electrophoresis on 0.8% agarose gels and transferred to Hybond N+ membranes. Genomic DNA was either prepared from cell lines using the SDS-proteinase K method, (Blumenfeld, A. et al., J. Med. Genet. 1993; 30:47-52) or directly from blood using the Chelex method (Walsh, P.S. et al., BioTech. 1991; 10:506-513). Blots were hybridized with probe DNAs radioactively labeled by random priming and visualized by autoradiography (Ozelius, L., et al., Neuron 1989; 2:1427-1434).

For typing simple sequence repeat polymorphisms, the method described by Weber, Am. J. Hum. Genet., supra, was used with the following modifications; PCR reaction volume was reduced to 10 μ l using 5-10 ng genomic DNA, 40 ng of each primer, and about 0.25 U Taq polymerase (Boehringer). In most cases α -³²P-dGTP (3,000 Ci/mmol, Amersham) was used as the labelled nucleotide. PCR conditions varied as has been previously described for the specific markers. Dried gels were subjected to autoradiography for 4-16 hours using Kodak X-OMAT AR film.

The following markers were used: D9S7 (Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Williamson, R. et al., Cytogenet. Cell Genet. 1991; 58:1190-1833), D9S15 (Kwiatkowski, D.J., et al., Genomics 1992; 12:229-240; Genome DataBase, Welch WH Medical Library,

- 22 -

Baltimore, MD 21205; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258, 67-86), D9S29 (Ozelius, L.J. et al., Genomics 1992; 14:715-720; Williamson, R. et al., Cytogenet. Cell
5 Genet. 1991; 58:1190-1833), D9S53 (Genome Data Base, Welch WH Medical Library, Baltimore, MD 21205; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Wilkie, P.J., et
10 al., Genomics 1992; 12:607-609), D9S58 (Kwiatkowski, D.J. et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86), D9S59 (Kwiatkowski, D.J.
15 et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720), D9S66 (Kwiatkowski, D.J. et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group,
20 Science 1992; 258:67-86), D9S105 (NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Wilkie, P.J. et al., Genomics 1992; 12:607-609), D9S106 (Wilkie, P.J. et al., Genomics 1992; 12:607-609), D9S109 (NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Furlong, R.A. et
25 al., Nucleic Acids Res. 1992; 20:925), D9S127 (NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Lyall, J.E.W. et al., Nucleic Acids Res. 1992; 20:925), HXB
30 (Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Ozelius, L., et al., Hum. Molec. Genet. 1992; 1:141; Povey,
35

- 23 -

° S. et al., Ann. Hum. Genet. 1992; 56:167-221), GSN (Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Williamson, R. et al., Cytogenet. Cell Genet. 1991; 58:1190-
5 1833), ABL (Kwiatkowski, D.J. et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-
10 86), and ASS (Kwiatkowski, D.J. et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86).

15 The LIPIN (v. 2.1) data management program was used for entry of marker phenotypes into a VAX4500 computer. Pairwise lod scores were calculated using MLINK (v. 3.5) and LINKMAP (V.4.9) (Lathrop, G.M. et al. Proc. Natl. Acad. Sci. USA 1984; 81:3443-3446). For multipoint analysis, the
20 loop in family 14 was broken, and only the portion of family 16 with two surviving affecteds was used. Consequently, the maximum multi-point lod score was slightly lower than the
25 maximum two-point score with D9S58. Autosomal recessive inheritance, complete penetrance, no rate of new mutations, and a gene frequency of 1/60 were assumed for familial dysautonomia.

30 The relative order of most of the markers has been established previously in both the Venezuelan reference pedigree and in th CEPH panel (Kwiatkowski, D.J. et al. Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics
35

- 24 -

• 1992; 14:715-720; NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Wilkie, P.J. et al. Genomics 1992; 12:607-609; Povey, S. et al., Ann. Hum. Genet. 1992; 56:167-221). To obtain accurate map distances for the multi-point
5 analysis, we genotyped D9S105 and D9S53 in the original 17 sibships of the Venezuelan reference pedigree (Tanzi, R.E. et al., Genomics 1988; 3:129-136. These data were analyzed in conjunction with previously typed markers (Kwiatkowski,
10 D.J. et al., Genomics 1992; 12:229-240; Ozelius, L.J. et al., Genomics 1992; 14:715-720) using the MAPMAKER program (version 1.0) (Lander, E.S. et al., Genomics 1987; 1:174-181). For comparison, we genotyped the CEPH panel for D9S59
15 and reanalyzed the previously reported data, NIH/CEPH Collaborative Mapping Group, Science 1992; 258:67-86; Wilkie, P.J. et al., Genomics 1992; 12:607-609). The distances used in the multi-point analysis (Figure 5) were
20 derived from the Venezuelan data set after error checking of apparent double recombinants. In both reference pedigree sets, the marker order was identical and the distances
25 between adjacent markers were similar.

The first DNA polymorphism that gave a significant positive lod score (Fig. 2) was HXB which is located on the long arm of chromosome 9 (Fig. 3). Table 1 provides the
30 oligonucleotide primer sequences for each polymorphism and the corresponding reference.

35

- 25 -

Table 1

	Marker	Oligonucleotide Primer Sequence*	Ref.
	HXB**	ATAGCCAAAGAGAGGTGCCC AGAGCCCTTCTGTCTTTTCC	1
5	D9S127**	CCCTCAAATTTGCTGTCTAT AGATTGATTGATACAAGGATTTG	2
	D9S58**	CCTGAGTAGCCGGGACTATA TAGGCAACACATCAAGATCCT	3
	D9S59**	AAGGGAATTCATCCCCTGCT TTACACTATACCAAGACTCC	3
10	ASS	GGTTGGCCTAAGAAAACCAT TGGGGAGCTATAAAAATGAC	3
	D9S66	CAGACCAGGAATGCATGAAG CACGGGCACACATGTATGC	3
15	D9S15	TAAAGATTGGGAGTCAAGTA TTCACTTGATGGTGGTAATC	3
	D9S53**	GCTGCATACTTTAACTAGC GGAATATGTTTTTATTAGCTTG	4
	D9S105**	GATCATATTGCTTACAACCC ACTTACTCATTAAATCTAGGG	4
20	D9S109**	GCACAGGCTGCAATATAGAC TTTACTGTATAAAAAGCTAATA	5
	D9S106**	ATTGTGTTGAAATTTGACCCCT CCAGGCTTATTTCCACACCT	4
25	ABL	TTTACACCTTCACCCAGAGA GGCTGTGTTTCAGTTAAACGT	3
	GSN**	CAGCCAGCTTTGGAGACAAC TCGCAAGCATATGACTGTAA	6

* Oligonucleotide primer sequences are listed 5' to 3'.

30 ** Markers linked to the familial dysautonomia gene.

1 Ozelius, L. et al., Human Mol. Genet. 1992; 1:141.

2 Lyall, J.E.W., et al., Nucl. Acids Res. 1992; 20:925.

3 Kwiatkowski, D.J., et al. Genomics 1992; 12:229-240.

4 Wilkie, P.J., et al., Genomics 1992; 12:604-609.

35 5 Furlong, R.A., et al., Nucl. Acids Res. 1992; 20:925.

6 Kwiatkowski, D.J., et al. Nucl. Acids Res. 1991; 19:967.

- 26 -

Based on the linkage results obtained with HXB, GT polymorphism analysis of chromosome 9 was performed using a panel of markers (See, Table 1) recently characterized in Kwiatkowski, D.J., et al., Genomics 1992; 12:229-240 (incorporated by reference); Lyall, J.E.W., et al., Nucl. Acid Res. 1992; 20(4):925 (incorporated by reference); Ozelius, L., et al., Hum. Mol. Genet. 1992; 1:141 (incorporated by reference); Wilkie, P.J., et al., Genomics 1992; 12:604-609 (incorporated by reference); Furlong, R.A. et al., Nucl. Acids. Res. 1992; 10:925 (incorporated by reference); Kwiatkowski, D. J. et al. Nucl. Acids Res. 1991; 19:967 (incorporated by reference) and D9S29 regular polymorphism (Williamson, R., et al., Cytogenet. Cell Genet. 1991; 58:1190-1833 (incorporated by reference)). Flanking markers on both sides of HXB were tested. Markers that were located closer to the centromere than HXB (e.g., D9S59, D9S58, D9S105, D9S127) gave higher lod scores, while those that were closer to the end (telomere) of the long arm (e.g., ASS) gave lower lod scores. See, Table 2.

The highest lod score was found with D9S58 (Kwiatkowski, et al., Genomics, supra) which has no recombinations between the marker and the disease status in all 26 familial dysautonomia families tested, and gave a lod score of 21.1 at zero distance. That means that D9S58 is located genetically at the same place as the familial dysautonomia gene with a ratio of $1:10^{21.1}$ in favor of linkage, while a ratio of $1:10^3$ is sufficient to prove

- 27 -

linkage, and the maximal lod score possibly available with the 26 FD families is about 23.5 ($1:10^{23.5}$ in favor of linkage). All other markers that were typed, gave lower lod scores than D9S58, and all of them also show recombination events between the marker and the familial dysautonomia gene in some of the families. The current lod scores on chromosome 9 markers that show some linkage to the familial dysautonomia gene are summarized in Fig. 2 and Table 2. Two flanking markers that are close to D9S58 are D9S59 (telomeric) and D9S127 (centromeric to D9S58). The closest flanking markers of those analyzed are D9S53 and D9S105. These markers were mapped genetically on both sides of D9S58 on large pedigrees, at distances of 4cM for D9S59 and about 15cM for D9S127, and were mapped physically to the same chromosomal region as D9S58. D9S58 was mapped to a chromosomal band q31 (Kwiatkowski, et al., Genomics, supra); D9S127 was mapped to the same band (Lyll, et al., Nucl. Acid Res., supra), and D9S59 to q31 or q32, (Kwiatkowski, et al., Genomics, supra) (Fig. 1).

Thus, genetic and physical data help to map the dysautonomia gene to chromosome 9q31, at the telomeric end of the band, and to a genetic region of about 20cM around D9S58, that correlates to about 20 million nucleotides. Markers D9S53 and D9S105 further restrict the location of the FD gene to within 10cM, i.e., 10 million nucleotides, around D9S58. Although D9S58 shows complete cosegregation with the familial dysautonomia gene in all dysautonomia

- 28 -

° families that were checked, it is not possible at this stage of research to claim that *D9S58* is located on top of the gene. More markers flanking *D9S58* at smaller genetic distances need to be found and tested in order to locate the
5 familial dysautonomia gene in a region small enough that will provide higher quality genetic tests for familial dysautonomia families (a region of 1-5 million nucleotides) and to specifically find the mutated gene. Narrowing down
10 the region in which the gene is located will lead to identifying/cloning of the familial dysautonomia gene as well as sequencing thereof. Further genetic analysis employing, for example, new polymorphisms flanking *D9S58* as
15 well as the use of cosmids, YAC (yeast artificial chromosomes) clones or mixtures thereof, can be employed in the narrowing down process and techniques such as PFGE
20 (pulsed field gel electrophoresis) or fingerprinting by Alu PCR. The next step in narrowing down will include cloning of the chromosomal region 9q31 including proximal and distal markers in a contig formed by overlapping cosmids.
25 Subsequent subcloning in cosmids, plasmids or phages will generate additional probes for more detailed mapping.

The next step of cloning the gene will involve exon trapping, screening of cDNA libraries, Northern blots
30 or rtPCR (reverse transcriptase PCR) of autopsy tissues from affected and unaffected individuals, direct sequencing of exons or testing exons by SSCP (single strand conformation

35

- 29 -

- ° polymorphism), RNase protection or chemical cleavage, or any other state-of-art technique.

Further localization of the FD gene to chromosome 9 was obtained as follows:

5

LINKAGE OF FD TO CHROMOSOME 9

Twenty-six families useful for linkage analysis were collected (Figure 1). The first marker locus that showed a significant positive lod score was *HXB* (Figure 3) in 9q32-q33 (\hat{z} -9.0 at $\hat{\theta}$ -0.04) (Table 2). Fourteen additional chromosome 9 markers, 9 mapping proximal to *HXB* and 5 mapping distal to *HXB*, were also tested (Table 2).

15

20

25

30

35

- 30 -

TABLE 2 - PAIRWISE LOD SCORES OF CHROMOSOME 9 MARKERS WITH FD

		RECOMBINATION FRACTION (Θ)							\hat{z}	$\hat{\Theta}$
	Marker	0.00	0.01	0.05	0.10	0.20	0.30	0.40		
5	<i>D9S15</i>	$-\infty$	-16.5	-5.5	-1.8	0.5	0.8	0.4	0.8	0.27
	<i>D9S29</i>	$-\infty$	3.8	5.5	5.3	3.8	2.1	0.6	5.6	0.06
	<i>D9S109</i>	$-\infty$	3.7	5.9	5.8	4.1	2.2	0.6	6.0	0.07
10	<i>D9S127</i>	$-\infty$	6.6	8.0	7.3	5.0	2.6	0.7	8.0	0.05
	<i>D9S53</i>	$-\infty$	10.4	10.8	9.5	6.3	3.2	0.9	11.0	0.03
	<i>D9S58</i>	21.1	20.6	18.3	15.5	9.9	5.0	1.5	21.1	0.00
15	<i>D9S105</i>	$-\infty$	13.8	12.8	11.0	7.2	3.7	1.1	13.8	0.01
	<i>D9S59</i>	$-\infty$	6.7	8.5	8.0	5.6	2.9	0.9	8.5	0.05
	<i>D9S106</i>	$-\infty$	10.1	10.5	9.4	6.3	3.3	0.9	10.7	0.03
20	<i>HXB</i>	$-\infty$	8.4	9.0	8.0	5.4	2.7	0.8	9.0	0.04
	<i>GSN</i>	$-\infty$	2.4	6.0	6.4	4.8	2.6	0.8	6.5	0.08
	<i>ABL</i>	$-\infty$	-7.9	-2.0	-0.1	0.7	0.5	0.1	0.7	0.21
25	<i>ASS</i>	$-\infty$	-14.9	-5.0	-1.6	0.4	0.6	0.2	0.6	0.26
	<i>D9S66</i>	$-\infty$	-17.6	-6.3	-2.4	0.1	0.4	0.2	0.5	0.29
	<i>D9S7</i>	$-\infty$	-6.7	-2.8	-1.4	-0.4	-0.1	-0.0	0.0	0.50

Restriction fragment length polymorphisms were used for *D9S29* and *D9S7*, while the remaining loci were typed using SSR polymorphisms. *ASS* was genotyped for both an RFLP and an SSR, and the results were haplotyped. Ten of the 15 markers tested detected significant linkage with FD although only *D9S58* showed no recombination events with the disease gene. These DNA markers all map to 9q22.3-q33 (Figure 3).

- 31 -

° DEFINITION OF FLANKING MARKERS

D9S58, which showed complete cosegregation with FD, was heterozygous in 59 of the 62 parents of affected children shown in Figure 1. the 1-lod unit confidence interval for the separation between FD and *D9S58* is 1.8 cM. For the purpose of prenatal diagnosis, however, use of a single marker is prone to potential error occasioned by rare crossover events with the disease gene. Thus, close flanking markers on either side of the FD gene are required to maximize the informativeness and accuracy of prenatal or carrier testing.

To define flanking loci, the phase of selected linked markers was determined in the FD families. The order of these loci as determined by combining data from the CEPH and Venezuelan reference pedigrees is: cen - (*D9S109*, *D9S127*)-*D9S53*-*D9S58*-*D9S105*-*D9S59*-*HXB*-tel (Figure 3). Recombination events in the FD families confirm this order and suggest that *D9S109* maps proximal to *D9S127*. Similarly, markers that did not map with significant odds in the reference pedigree data could be positioned tentatively as follows: *D9S29* proximal to *D9S109* and *D9S106* within the interval *D9S59*-*HXB*. Figure 4 shows examples of recombination events detected within the FD pedigrees. In Figure 4A, recombination was detected between *D9S53* and *D9S105*, with FD segregating with the telomeric markers. Unfortunately, in this instance the mother was homozygous at *D9S58* limiting the assignment of FD to a position distal to

- 32 -

• D9S53. Figure 4B displays two additional simple crossovers that place the FD gene proximal to D9S105 and distal to D9S53, respectively. These, and additional crossovers (not shown) are consistent with D9S53 and D9S105 being the closest flanking markers.

To provide a statistical basis supporting the definition of flanking markers, we performed multipoint linkage analysis. FD was analyzed relative to four firmly mapped marker loci: D9S53, D9S58, D9S105 and D9S59 (Figure 5). The genetic distances between the markers were calculated from the Venezuelan references pedigree. (Tanzi, R.E. et al., Genomics 1988; 3:129-136). This analysis firmly positioned FD coincident with D9S58, between D9S53 and D9S105. A localization within this interval was favored by more than 10⁵:1 over any other interval, confirming D9S53 and D9S105 as flanking markers for genetic diagnosis.

LINKAGE DISEQUILIBRIUM WITH FD

The restriction of FD to individuals of Ashkenazi Jewish ancestry suggests the possibility of a founder effect in which most or all affected alleles share a common origin. Consequently, we examined the closely linked markers for evidence of allele association. Marker genotypes were obtained for 353 different FD chromosomes from the 26 linkage families and 148 families with single affected individuals. Four marker loci, D9S58, D9S59, D9S105 and D9S106, yielded χ^2 values significant at $p < 0.01$. The

- 33 -

- allele association with FD at D9S58 and D9S105 was particularly striking (Table 3).

5

10

15

20

25

30

35

- 34 -

TABLE 3 -- LINKAGE DISEQUILIBRIUM OF FD WITH *D9S58* AND *D9S105*

	MARKER ALLELE	PCR PRODUCT SIZE (bp)	NUMBER OF CHROMOSOMES		
			CONTROL OBSERVED	FD EXPECTED	FD OBSERVED
5	<i>D9S58</i>				
	1	151	1	1	0
	2	149	1	1	0
	3	147	12	16	1
	4	145	5	7	0
	5	143	3	4	4
10	6	141	3	4	0
	7	139	10	14	2
	8	137	23	31	4
	9	135	22	30	10
	10	133	6	8	1
	11	131	16	22	3
15	12	129	20	27	9
	13	127	36	49	37
	14	125	16	22	3
	15	123	18	24	2
	16	121	18	24	14
	17	119	2	3	1
20	18	117	14	19	256
	19	115	16	22	2
	20	113	15	20	2
	21	105	2	3	1
	22	101	<u>1</u>	<u>1</u>	<u>1</u>
			260	353	353
			$X^2 = 3142$ 15 D.F.* P < 0.0001		
25	<i>D9S105</i>				
	1	203	6	9	4
	2	201	13	19	12
	3	199	19	27	18
	4	197	34	49	20
	5	195	29	42	21
	6	193	13	19	9
	7	191	24	35	37
	8	189	64	93	186
	9	187	11	16	4
30	10	183	<u>2</u>	<u>3</u>	<u>0</u>
			215	311	311
			$X^2 = 147$ 8 D.F.* P < 0.0001		

* For *D9S58* and *D9S105* classes 1, 2, 5, 6, 17, 21 and 22 and classes 1 and 10, respectively, were clustered.

- 35 -

D9S58 displayed 22 alleles in a collection of 260 control chromosomes from the Ashkenazi Jewish population (Table 3). Eighteen of these alleles were seen on FD chromosomes, but the "18" allele (117 bp) was strikingly overrepresented. Of the 353 FD chromosomes available, 256 or 73% displayed an "18" allele for *D9S58*. This compares with a frequency of 5% (14 of 260) in the control Ashkenazi Jewish population. The allele association with FD was highly significant ($\chi^2=3142$, 15 d.f. (based on pooling classes with expected values less than 5), $p<0.0001$).

D9S105, located about 3 cM from *D9S58*, also displayed significant linkage disequilibrium with FD ($\chi^2=147$, 8 d.f., $p<0.0001$). *D9S105* possessed 10 alleles in the control population (Table 3). Allele "8" (189 bp), the most common allele in the control population (30%) was overrepresented in FD (60%).

As expected, the most frequent haplotype of *D9S58* and *D9S105* on FD chromosomes was "18,8" (54%). This haplotype was rare in control Ashkenazi Jews, representing just 2.5% of control chromosomes. Since the carrier frequency for FD is estimated at 3.3%, many of the "18,8" chromosomes present in the normal population may reflect FD chromosomes present in undetected carriers.

χ^2 values for *D9S59* and *D9S106* were 18.1 (4 d.f.; $p < 0.005$) and 17.3 (6 d.f.; $p < 0.01$), respectively. The next markers proximal and distal (Figure 3), *D9S53* and *HXB*, showed no allele association with FD (data not shown).

- 36 -

Flanking markers on both sides of the familial dysautonomia gene combined with *D9S58*, or a number of well-positioned markers that cover the chromosomal region (q31) carrying the disease gene, can give a high probability of affected or non-affected chromosomes in the range of 80-90% accuracy, depending on the informativeness of the markers used and their distance from the disease gene. Using the current markers linked to the familial dysautonomia gene, or preferably closer flanking markers when they are identified (using the above methods), a genetic test for families with familial dysautonomia-affected member is provided for both prenatal diagnosis and carrier test in healthy siblings. Subsequent delineation of even more closely linked markers which may show strong disequilibrium with the disorder, or identification of the defective gene, could also allow screening of the entire at-risk population to identify carriers, and potentially reduce the incidence of new cases of familial dysautonomia. Such closer markers, for example, *D9S53* and *D9S105* have now been identified and further map the location of the FD gene to chromosome region 9q31-q33.

The method lends itself readily to the formulation of kits which can be utilized in diagnosis. Such a kit would comprise a carrier being compartmentalized to receive in close confinement one or more containers wherein a first container may contain DNA containing coding sequences which may be used to identify a given polymorphism, e.g. an SSR. A second container may contain a different set of sequences

- 37 -

- coding for a second SSR, and so on. Other containers may contain reagents useful in the detection of the labelled probes, such as enzyme substrates. Still other containers may contain restriction enzymes, buffers, and the like.

5 It will be obvious to those skilled in the art to which the invention pertains, that various changes and modifications may be made without departing from the scope of the invention defined by the claims.

10

15

20

25

30

35

- 38 -

WE CLAIM:

1. A method for detecting the presence in a subject of a gene associated with familial dysautonomia which comprises:

5 analyzing human chromosome 9 of the subject for a DNA polymorphism linked to the familial dysautonomia gene.

10 2. The method according to claim 1, wherein the polymorphism is located on the long arm of human chromosome 9.

15 3. The method according to claim 2, wherein the polymorphism is located on the q31 band of the long arm of human chromosome 9.

20 4. The method according to claim 2, wherein the polymorphism is selected from the group consisting of D9S53, D9S58, D9S59, D9S105, D9S127 and HXB.

25 5. The method according to claim 2, wherein the polymorphism is located about 20 cM around D9S58.

30 6. The method according to claim 5, wherein the polymorphism is located about 10 cM around D9S58.

35

- 39 -

7. The method according to claim 6, wherein the polymorphism is *D9S58*.

8. The method according to claim 2, wherein the polymorphism is located about 20 cM around *D9S127*.

9. The method according to claim 8, wherein the polymorphism is located about 10 cM around *D9S127*.

10. The method according to claim 9, wherein the polymorphism is *D9S127*.

11. The method according to claim 2, wherein the polymorphism is located on the chromosome between *D9S59* and *D9S127*.

12. The method according to claim 4, wherein the polymorphism is located on the chromosome between *D9S53* and *D9S105*.

13. The method according to claim 2, wherein the analysis is carried out by:

- (a) amplifying the polymorphism;
- (b) separating the amplified polymorphism to generate a polymorphism pattern;
- (c) correlating the presence or absence of the polymorphism with the respective

- 40 -

presence or absence of the affected gene by comparing a corresponding polymorphism pattern for family members showing segregation between the familial dysautonomia gene and the polymorphism.

14. The method according to claim 12, wherein the polymorphism is detected by autoradiography.

15. The method according to claim 12, wherein the polymorphism pattern of the subject is compared to the corresponding polymorphism pattern for each parent of the subject which are unaffected by familial dysautonomia disease and a family member affected by familial dysautonomia disease.

16. A method for detecting the presence of a gene associated with familial dysautonomia in a subject comprised of determining a maternal polymorphism linked to a gene associated with familial dysautonomia; determining a paternal polymorphism linked to a gene associated with familial dysautonomia; typing the subject to determine the maternal polymorphism and paternal polymorphism; linking the distribution of the maternal polymorphism and paternal polymorphism with familial dysautonomia; and determining if

35

- 41 -

- the subject has the polymorphism associated with familial dysautonomia.

5 17. The method according to claim 16, wherein the polymorphism is located on the long arm of human chromosome 9.

10 18. A method for detecting the presence of a familial dysautonomia gene in a subject comprising typing blood relatives of a subject for a polymorphism located on the long arm of human chromosome 9 linked to the familial dysautonomia gene; and analyzing DNA from the subject for
15 the presence of the polymorphism.

20 19. The method according to claim 17, wherein the polymorphism is located within 20 cM around D9S58.

25 20. The method according to claim 17 wherein the polymorphism is located between D9S53 and D9S105.

 21. The method according to claim 18 wherein the polymorphism is located in the chromosome region 9q31-q33.

30 22. The DNA sequence according to claim 23 wherein the DNA consists essentially of the DNA sequence of chromosome region 9q31-q33.

35

- 42 -

23. A substantially pure DNA sequence containing the familial dysautonomia gene consisting essentially of the DNA sequence of the long arm of human chromosome 9 located between *D9S59* and *D9S127*.

5

24. The substantially pure DNA sequence according to claim 23 wherein the DNA sequence consists essentially of the DNA sequence located between *D9S53* and *D9S105*.

10

25. A kit for detecting the presence of the FD gene in an individual, the kit comprising at least one nucleic acid probe having a sequence that can identify a polymorphism linked to the familial dysautonomia gene.

15

26. The kit according to claim 25 wherein the nucleic acid sequence that can identify a polymorphism selected from the group consisting of *D9S53* and *D9S105*.

20

25

30

35

1/6

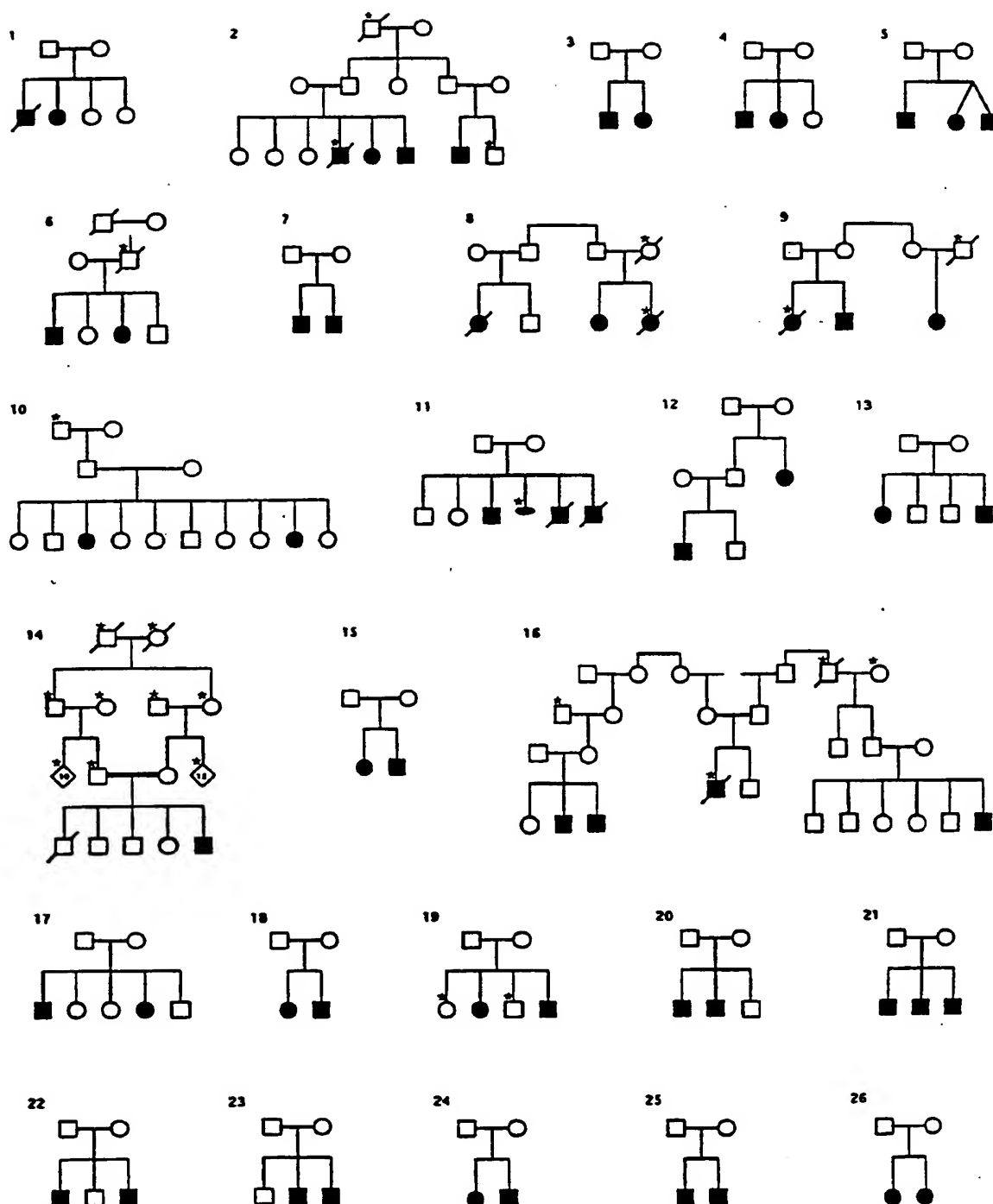


FIG. 1

SUBSTITUTE SHEET

2/6

Lod Scores Between Dysautonomia and Chromosome 9 Markers

Marker	Recombination Value							
	0.00	0.05	0.10	0.20	0.30	0.40	θ	z
D9S15	$-\infty$	-5.54	-1.76	0.53	0.76	0.36	0.266	0.81
D9S109	$-\infty$	5.54	5.42	3.85	2.04	0.60	0.066	5.62
D9S29	$-\infty$	5.56	5.35	3.82	2.04	0.60	0.063	5.59
D9S127	$-\infty$	8.01	7.21	4.82	2.44	0.68	0.040	8.05
D9S58	18.48	15.93	13.39	8.47	4.22	1.23	0.000	18.48
D9S59	$-\infty$	6.08	6.05	4.39	2.36	0.74	0.070	6.21
HXB	$-\infty$	5.99	5.89	4.27	2.30	0.70	0.068	5.91
GSN	$-\infty$	6.09	6.39	4.79	2.62	0.83	0.085	6.45
ASS	$-\infty$	-5.07	-1.64	0.41	0.55	0.20	0.303	0.56

FIG. 2

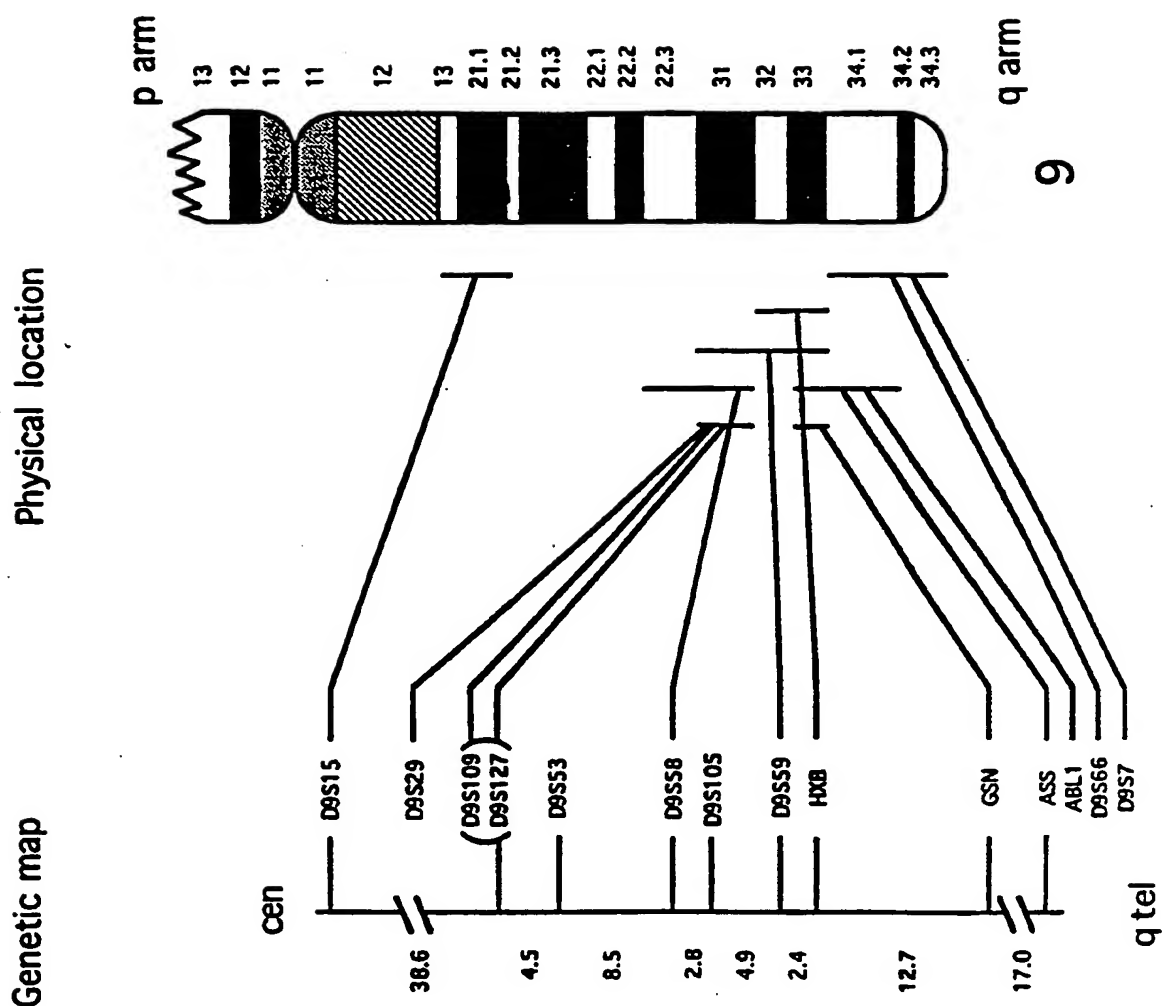


FIG. 3

4/6

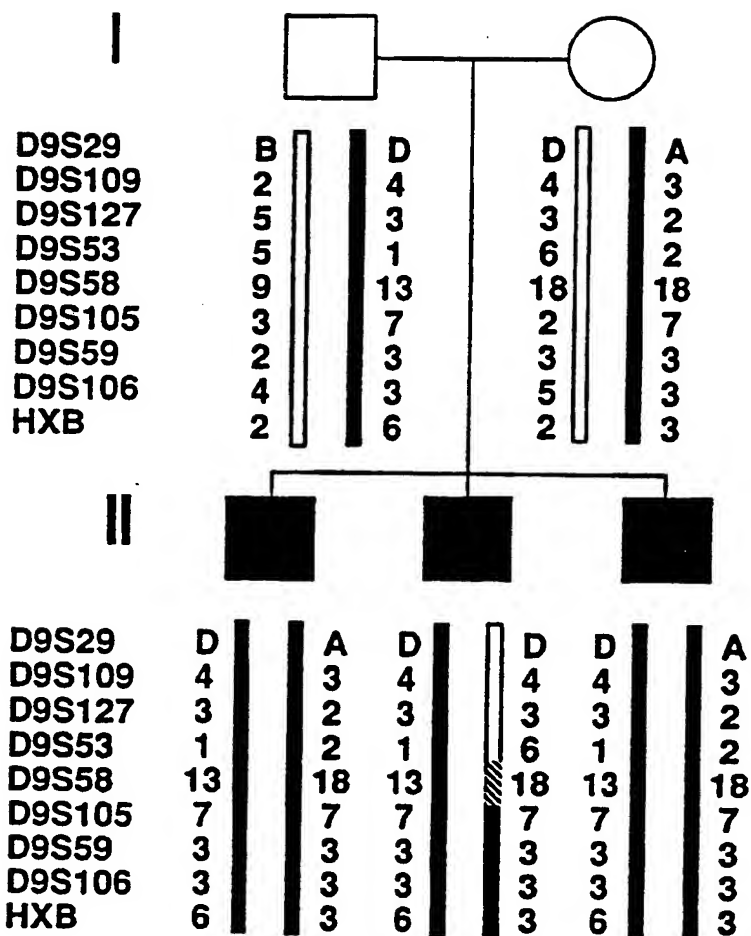


FIG. 4A

SUBSTITUTE SHEET

5/6

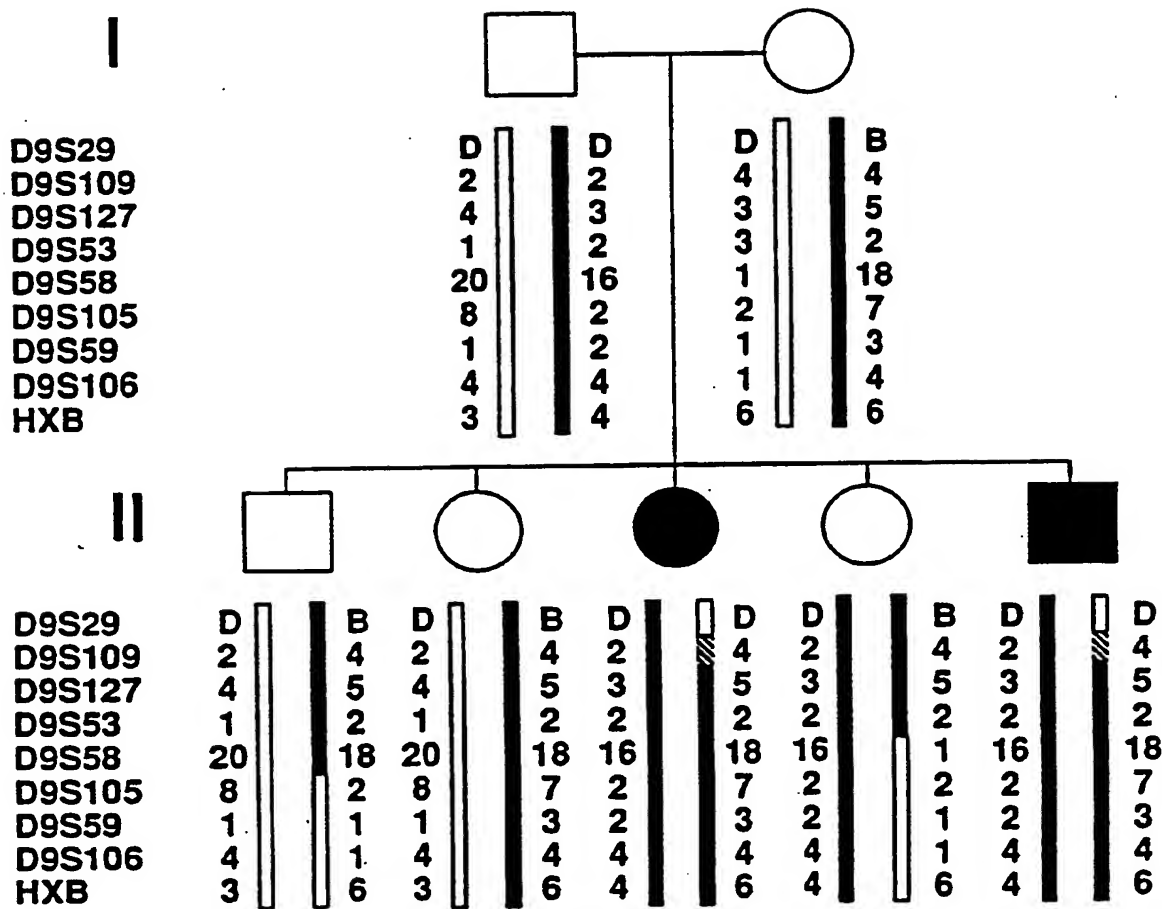


FIG. 4B

SUBSTITUTE SHEET

6/6

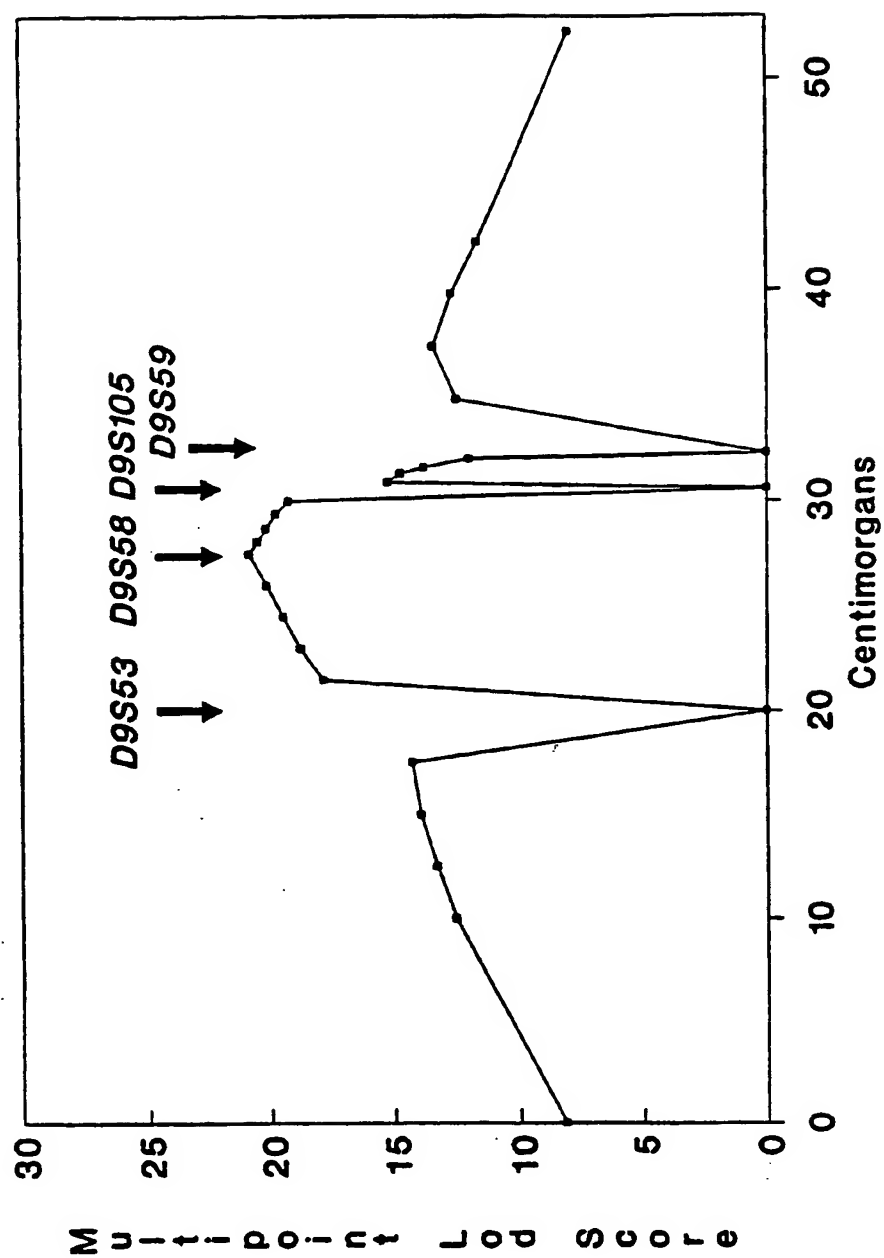


FIG. 5

SUBSTITUTE SHEET